UNIVERSITI PUTRA MALAYSIA

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FPSK(M) 2005 4

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

Jun 2005

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

MOLECULAR CLONING AND GENE EXPRESSION ANALYSIS OF THE PHOSPHOLIPASE B GENES OF NON-*ALBICANS CANDIDA* SPECIES

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CHEANG PEY SHYUAN

June 2005

Chairman : Chong Pei Pei, PhD

Faculty : Medicine and Health Sciences

Phospholipase B (*PLB*) is known to be a virulence factor of *Candida albicans*. It hydrolyzes phospholipids and deranges constituents of host cell membranes which are likely to be involved in host cell invasion. The aims of the study are to clone and sequence the *PLB* genes of non-*albicans Candida* species and to assess the time-dependent *PLB* gene expression of *C. albicans*, *C. krusei* and *C. tropicalis* under the hyphal induction condition. Three pairs of degenerate primers designed from the conserved regions of *PLB*1 genes of *C. albicans, Saccharomyces cerevisiae* and other fungi were used to amplify DNA fragments of the *PLB* genes from *C. krusei, C. parapsilosis* and *C. tropicalis* by the polymerase chain reaction (PCR) approach. Amplicons of approximately 510 bp were successfully obtained from the ATCC type strains of *C. krusei*, *C. parapsilosis* and *C. tropicalis* as well as a *C. tropicalis* clinical blood isolate, by using the first primer pair that targeted the 5' proximal end region of the putative *PLB* gene. The second and third pairs of degenerate primers amplified fragments corresponding to the 3' proximal end region of the putative *PLB* genes from the ATCC strains as well as clinical isolates of *C. parapsilosis* and *C.*

tropicalis. These PCR products were cloned into *E. coli* vectors and subjected to DNA sequencing. The deduced amino acid sequences of these cloned partial open reading frames (ORFs) exhibited significant homology of about 60-70% identity with known fungal *PLB* genes. Sequence analysis showed that these putative *PLB* homologues and five other known *PLB*s are contained in a cluster of *PLB* family of proteins in which the amino acid sequences are more conserved towards the carboxyl terminus. Attempts to clone the full length gene sequences of the non-*albicans* *Candida* using inverse PCR and RACE (Rapid Amplification of cDNA Ends) were futile. Using a simple phospholipase assay, varying levels of enzyme activity for the extracellular phospholipase was demonstrated in cultures of *C. albicans, C. krusei,* and *C. tropicalis* ATCC strains as well as *C. parapsilosis* and *C. tropicalis* clinical blood isolates. *C. albicans* produced significantly higher extracellular phospholipase relative to the other *Candida* species used in this study. *C. glabrata and C. parapsilosis* ATCC cultures showed undetectable extracellular phospholipase activity. In order to assess the *PLB* gene expression at mRNA level, semi-quantitative RT-PCR approach was undertaken. The *PLB* gene expression time course analysis showed that there were significant differences in gene expression level over the 24 hours duration for cultures of *C. albicans*. Both the yeasts and hyphae forms expressed *PLB* gene at the mRNA level. *PLB* gene expression was undetectable in *C. krusei*, *C. tropicalis* ATCC and *C. tropicalis* clinical blood isolates in both the yeast and pseudohyphae forms. The results of this study strongly suggest that *PLB* is not a significant virulence determinant of non-*albicans* species. However, the data generated here would provide the vital groundwork for elucidating the instrinsic functional role of *PLB*s in the virulence and pathogenesis of the *albicans* and non-*albicans* *Candida* species.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGKLONAN MOLEKULAR DAN ANALISIS PENGEKSPRESAN GEN FOSFOLIPASE B UNTUK SPESIS BUKAN-*ALBICANS CANDIDA*

Oleh

CHEANG PEY SHYUAN

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Pengerusi : Chong Pei Pei, PhD

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Fosfolipase B telah dikenalpasti sebagai satu factor virulen bagi *Candida albicans*. Ia menghidrolisiskan fosfolipid dan mengganggu susunan membran sel perumah, mencadangkan bahawa fosfolipase B mengambil bahagian dalam penyerangan masuk ke dalam sel perumah. Kajian ini bertujuan untuk mengklon dan menjujuk gen *PLB* dari spesis bukan *Candida albicans* dan juga meneliti pengekspresan gen *PLB* yang bergantung kepada tempoh masa apabila didedahkan kepada keadaan induksi hifa. Tiga pasangan primer degenerat yang direkacipta dari lokasi terpelihara jujukan nukleotid *PLB*1 *C. albicans*, *Saccharomyces cerevisiae* dan fungi lain telah diguna untuk amplifikasi fragmen gen *PLB* daripada *C. krusei*, *C. parapsilosis* dan *C. tropicalis* melalui pendekatan reaksi rantaian polymerase (PCR). Amplikon dengan kira-kira 510 pasangan bes berjaya diperolehi daripada *C. krusei*, *C. parapsilosis*, *C. tropicalis* strain ATCC dan juga *C. parapsilosis* dan *C. tropicalis* isolat darah klinikal dengan menggunakan pasangan primer degenerat pertama yang mengecam lokasi hujung proksimal 5' gen *PLB*. Pasangan primer degenerat kedua dan ketiga pula mengamplifikasikan fragmen yang sejajar dengan lokasi hujung proksimal 3' gen *PLB* putatif daripada strain ATCC dan juga isolat darah klinikal *C. parapsilosis* dan *C. tropicalis*. Hasilan PCR diklonkan ke dalam vektor *E. coli* dan digunakan untuk penjujukan DNA. Jujukan deduksi asid amino bagi sebahagian ORF klon

telah menunjukkan homologi signifikan kira-kira 60-70% identiti dengan gen *PLB* fungi yang telah dikenalpasti. Analysis jujukan menunjukkan bahawa homolog *PLB* putatif dan lima gen *PLB* yang telah dikenalpastikan jujukan adalah terkandung dalam satu kelompok protein keluarga *PLB* dan jujukan asid amino adalah lebih terpelihara semakin menuju ke terminal karbosil. Percubaan-percubaan untuk mendapatkan jujukan penuh gen *PLB* bagi spesis bukan *albicans* telah dijalankan dengan menggunakan teknik inversi PCR dan RACE (Amplifikasi Pantas untuk Hujung cDNA) tetapi tidak berjaya. Dengan menjalankan eksperimen fosfolipase yang ringkas, paras aktiviti enzim fosfolipase ekstrasel yang berbeza-beza telah ditunjukkan dalam kultur *C. albicans*, *C. krusei*, *C. tropicalis* strain ATCC dan juga isolat darah klinikal *C. parapsilosis* dan *C. tropicalis*. Paras perembesan fosfolipase ekstrasel bagi *C. albicans* adalah lebih tinggi jika berbanding dengan *Candida* yang lain dalam kajian ini. Aktiviti fosfolipase ektrasel bagi kultur *C. glabrata* dan *C. parapsilosis* ATCC tidak dapat dikesan sama sekali. Untuk mengaji pengekspresan gen *PLB* pada paras mRNA, pendekatan kuantitatif-separa RT-PCR telah diambil. Analysis tempoh masa pengekspresan gen *PLB* menunjukkan bahawa terdapat perbezaan signifikan paras pengekspresan gen sepanjang tempoh 24 jam untuk *C. albicans*. Kedua-dua yis dan hifa menunjukkan pengekspresan gen *PLB*. Pengekspresan gen *PLB* tidak dapat dikesan daripada strain ATCC *C. krusei*, *C. tropicalis* dan juga isolat darah klinikal *C. tropicalis* sepanjang tempoh 24 jam untuk yis dan hifa palsu (pseudohyphae). Keputusan-keputusan yang diperolehi daripada kajian ini mencadangkan bahawa *PLB* bukan penentu virulen yang signifikan bagi spesis bukan *albicans*. Walaubagaimanapun, data yang telah diperolehi merupakan asas kerja yang penting bagi meneliti fungsi intrinsik *PLB* dalam virulen dan mekanisme patogenik spesis untuk *C. albicans* dan juga bukan *albicans*.

ACKNOWLEDGEMENTS

My special thanks go to Dr. Chong Pei Pei, the research project main supervisor, for the consistent, invaluable assistance, guidance and funding; understanding and kindness she has given and shown to me during the course of the scientific research. My acknowledgements also go to Professor Seow Heng Fong and Associate Professor Dr. Mariana Nor Shamsudin, the project co-supervisors for contributing their ideas, constructive criticisms and suggestions to improve the many aspects of the experiments; and Associate Professor Dr. Mirnalini Kandiah who helped me in the statistical test and analysis.

To the Biochemistry, Immunology, Microbiology laboratories, and media room technicians and assistants, namely, Mr. Rizal, Mr. Anthonysamy, Mr. Rahman, Mr. Tung and others, thank you for providing scientific apparatus and facilities along the path of the research.

With me always are the colleagues from Prof. Seow's and Dr. Chong's laboratories; respectively they are Ban (from China), Maha, Khor, Tina Ong, Hwen Yee, Pei Ching, and Janet; Won Fen, Chee Long, David, Phelim and Lee Yean. I thank you for the help and consideration shown to me throughout the years.

Last but not least, loving kindness and appreciation to my parents, teachers, family members, relatives, and the Sri Serdang house residents, to whom I compliment your sincere encouragement and support during the three years duration of the research. Thank you very much.

I certify that an Examination Committee met on 2nd June 2005 to conduct the final examination of Cheang Pey Shyuan on her Master of Science thesis entitled "Molecular Cloning and Gene Expression Analysis of the Phospholipase B Genes of Non-*Albicans Candida* Species" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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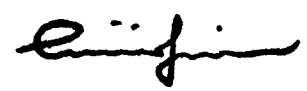

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Date: 08 SEP 2005

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

CHEANG PEY SHYUAN

Date: 18 AUGUST 2005

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency
ATCC	American Type Collection Culture
ATP	Adenosine triphosphate
bp	Base pair
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CIP	Calf intestinal phosphatase
CO_2	Carbon dioxide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
EBI	European Bioinformatics Institute
EDTA	Ethylenediamine tetraacetic acid
e.g.	*exempli gratia* (Latin), for example
EIA	Enzyme immunoassay
et al.	*et alii* (Latin), and others
GSP	Gene specific primer
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
Hrs	Hours
ICU	Intensive care unit
IPCR	Inverse PCR
IPTG	Isopropyl-1-thio-D-galactopyranoside
ITS	Internal transcribed spacer
kb	Kilo base
kDA	Kilo dalton
LA-PCR	Ligation anchored PCR
LB broth	Luria-Bertani broth
M	Molar
$MgCl_2$,	Magnesium chloride

$MgSO_4$	Magnesium sulphate
min	Minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
NaCl	Natrium chloride
NCBI	National Center for Biotechnology Information
°C	Degree celcius
OD_{550}	Optical density at 550 nanometer
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PL	Phospholipase
PLA	Phospholipase A
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
pmoles/µl	Picomoles per microlitre
RACE	Rapid amplification of cDNA end
RE	Restriction enzyme
RL-PCR	Reverse ligation-mediated PCR
RLM-RACE	RNA ligase-mediated RACE
rpm	Revolution per minute
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RT-PCR	Reverse transcription-PCR
SAP	Secreted aspartyl proteinase
sec	Second
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud dextrose broth
SDS	Sodium dodecyl sulphate
SLIC	Single-stranded ligation to single-stranded cDNA ends
TAE	Tris-acetate EDTA
TAP	Tobacco acid pyrophosphatase

Taq	*Thermus aquaticus*
µg/ml	Microgramme per millilitre
µl	Microlitre
u/µl	Unit per microlitre
V	Volt
v/v	Volume per volume
w/v	Weight over volume
Y	Yeast
Y-H	Yeast-hypha
YNB	Yeast-nitrogen base
YPD	Yeast-peptone-dextrose

CHAPTER 1

INTRODUCTION

1.1 Background Study

Candida species are yeasts that are usually relatively benign commensals of human. The ability of *Candida* to become virulent is primarily determined by the immune state of the host (Lortholary and Dupont, 1997; Ashman, 1998). As *Candida* species are opportunistic organisms, they cause chronic-to-acute infections when the host's immune system is deteriorated. There are many clinical manifestations of candidal infections, such as oropharyngeal candidiasis, vulvovaginal candidiasis, chronic mucocutaneous candidiasis, *Candida* endocarditis and meningitis, as well as a potentially lethal and increasingly common nosocomial bloodstream infection known as candidemia (Wenzel, 1995). Growing pools of individuals who undergo cancer chemotherapy, organ transplantation, or infection with human immunodeficiency virus (HIV) are susceptible to such systemic infections (Corner and Magee, 1997).

Candida species ranked as the fourth most important pathogens causing bloodstream infections, and *C. albicans* is the major fungal agent isolated during systemic infections in United States (Edmond *et al.*, 1999; Pfaller *et al.*, 2000). Recently, there has been an important shift in the type of *Candida* infections – away from *C. albicans* – to more treatment-resistant, non-*albicans* varieties. In 1990, nearly 80% of the reported candidemia was attributed to *C. albicans*, but in an epidemiologic study that investigated the distribution of bloodstream isolates from patients in the

ICUs, *C. albicans* was the causative pathogen in just 48% of *Candida* nosocomial bloodstream infections, with the remaining *Candida* infections attributed to *C. glabrata* (24%), *C. tropicalis* (19%) and *C. parapsilosis* (7%) (Rangel *et al.*, 1999). The same scenario was also reported in Malaysia, where non-*albicans Candida* was the most common *Candida* species isolated from blood, respiratory system, urine and skin. From 1997 to October 1999, among the 102 *Candida* biotypes isolated from blood cultures, 51% of the *Candida* species were *C. parapsilosis,* 25.5% were *C. tropicalis* whereas *C. albicans* only made up 11.8%. 6.9% were *C. rugosa,* 3.8% were *C. glabrata* and 1% was *C. guilliermondii* (Ng *et al.*, 2000). Patients with candidemia have the shortest survival prospects; Morgan *et al.* (2005) reported in a study of hospital-acquired candidemia, the mortality attributable to candidemia ranged between 19% and 24%.

Along with an increase in the overall incidence of candidiasis, there is a shift towards infections by inherently azole drug-resistant species, especially *C. glabrata* and *C. krusei.* These two species are known to be innately less susceptible to the commonly used azole antifungal agents such as fluconazole and ketoconazole (Marr *et al.*, 2000). Furthermore, *C. tropicalis* and *C. parapsilosis* are inherently resistant or can quickly develop resistance to polyenes and azole drugs; thus, their increasing prevalence may contribute to the steady increase in nosocomial bloodstream infections seen over the past decades (Bodey, 1993; Rex *et al.*, 1995; Nguyen *et al.*, 1996). Therefore, detection and diagnosis of invasive candidiasis to the species level is crucial so that appropriate treatment can be prescribed.

Early detection of fungal infection has a great impact on the clinical outcome of invasive candidiasis. Unfortunately, using conventional methods of morphologic and metabolic characteristics may take three to five days or even longer for identification of fungal isolates. Due to low positivity of blood cultures, diagnosis of haematogenous candidiasis has been problematic (Pizzo and Walsh, 1990).

Although various laboratory tests based on detection of *Candida*-specific antibodies, antigens, or metabolites have been developed, these tests are lacking in specificity or sensitivity, besides being time-consuming. Moreover, these tests fail to clearly discriminate between the infecting *Candida* species, which is crucial information to initiate specific antifungal therapy since several non-*albicans Candida* species are known to be resistant to commonly used antifungal drugs (Johnson *et al.*, 1995; Walsh and Chancock, 1997; Posteraro *et al.*, 2000).

In recent molecular diagnostic approaches, candidal DNA amplification using polymerase chain reaction (PCR) together with enzyme immunoassay (EIA) have been able to detect a variety of *Candida* species in a rapid, sensitive and specific manner. Many publications have described the development of PCR-based methods for detecting candidemia and other fungal infections. However, the methods are not widely utilised due to the problems of cross contamination with nested-PCR, the requirement of technical expertise and labour intensiveness. Hence there is a critical need to improve and refine the existing diagnostic methods.

Due to the technological advancement in molecular pathogenesis research methodologies, it is now possible to identify and to validate potential molecular

targets that are essential for fungal virulence. The identification of unique genes, differential gene expression and the signaling pathways, important to the pathobiology of fungi, can translate into molecular targets for new antifungal design or diagnostic tools (Wills *et al.*, 2000).

In order to invade the host tissues, microbial cells need to damage and further penetrate the outer envelope. This process is most probably mediated by either physical or enzymatic means or a combination of the two. Since cell membranes are made up of lipids and proteins, therefore hydrolytic enzymes such as proteinases, which hydrolyze peptide bonds, and phospholipases, which hydrolyze phospholipids that can be induced to destroy or derange constituents of host cell membranes, leading to lysis, are likely to be involved in host cell invasion. There are evidence implicating the role of phospholipases in host cells penetration, injury and lysis by microorganisms such as *Rickettsia rickettsii*, *Toxoplasma gondii*, *Entamoeba histolytica* and *Candida albicans* (Ghannoum, 2000).

The role of candidal phospholipases as virulence factors was reported and evidenced by the more significant production of extracellular phospholipase activity in invasive strains compared to the commensal or non-invasive strains (Ibrahim *et al.*, 1995). In addition, variation of phospholipase activities among *C. albicans* strains have been reported and only strains which have high level of phospholipase activity were shown to be able to cross the bowel wall and undergo subsequent hematogenous dissemination and tissue invasion (Ghannoum, 2000).

In *C. albicans*, various phospholipases, i.e. phospholipase A, phospholipase B,

phospholipase C, phospholipase D, lysophospholipase, and lysophospholipase-transacylase have been identified. Among all these phospholipases, only phospholipase B1 has been suspected of its role as virulence factor in animal model of candidiasis (Ghannoum, 2000). As the role of candidal phospholipase B is strongly associated with pathogenesis particularly during the progression of the invasive infection, this virulent determinant can be used as a diagnostic tool or as the target for the design of antifungal agents.

However, genomic and functional studies of yeast phospholipases have so far been confined to *Saccharomyces cerevisiae* and *C. albicans*. Research efforts on other virulent and emerging species such as *C. krusei, C. parapsilosis* and *C. tropicalis* are minimal. The key difference between *C. albicans* and the other major *Candida* species is that *C. albicans* can produce hyphae under certain conditions whereas the latter species do not produce hyphae under the same conditions, but rather pseudohyphae. Besides, various studies have raised a concern that extensive usage of azole-antifungal agents have resulted in the emerging of azole-resistant strains and caused a shift in the prevalence of *C. albicans* species towards non-*albicans* species. Moreover, the involvement of phospholipases particularly phospholipase B (PLB) in invasive infection in the non-*albicans* species has not yet been investigated. Hence this project was undertaken to firstly clone the *PLB* genes of these species and secondly to attempt to unravel the role of PLBs in the pathogenesis of these species.